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Oligonucleotide Characterization by Bio LC and Q-TOF

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Introduction

Synthetic oligonucleotide is a fast-growing new modality in recent years. They are present in various sizes and forms and are often chemically modified to enhance product effect, stability, specificity, and delivery efficiency. The most common quality attributes of synthetic oligonucleotides are their mass, purity, contaminating impurities, and sequence.

Characterization of oligonucleotides requires robust analytical instrumentation and methods as well as ease-of-use data analysis tool. Agilent 1290 Infinity II Bio LC System is made of biocompatible material for use with challenging mobile phase conditions in biopharma applications. Biocompatibility mitigates nonspecific sample binding to flow path and it ensures the integrity of biomolecules and robustness of the system. The Target Plus Impurities (TPI) and Sequence Confirmation (SC) workflows in Agilent MassHunter BioConfirm software have already been introduced in two separate application notes [1,2]. In this study, both workflows were carried out to characterize two oligonucleotide samples using Agilent 1290 Infinity II Bio LC System coupled to Agilent 6545XT AdvanceBio LC/Q-TOF (Figure 1).

Experimental

Sample preparation

Oligonucleotide (RNA) Resolution Standard was obtained from Agilent. An 18-mer antisense oligonucleotide (ASO) and 28-mer aptamer were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) with standard desalting purification. Sample details are listed in Table 1.

Name	Length	Sequence
ASO	18-mer	/52MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErC/*/i2MOErT/*/i2MOErT/*/i2MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErT/*/i2MOErA/*/i2MOErA/*/i2MOErT/*/i2MOErG/*/i2MOErC/*/i2MOErT/*/i2MOErG/*/32MOErG/
Aptamer	28-mer	/52FC/mGmGrArA/i2FU//i2FC/mAmG/i2FU/mGmAmA/i2FU/mG/i2FC//i2FU//i2FU/mA/i2FU/mA/i2FC/mA/i2FU//i2FC//i2FC/mG/3InvdT/

Table 1. Oligonucleotide sample sequences in the 5' to 3' direction.

Sample Analysis

LC/MS analyses were conducted on a 1290 Infinity II Bio LC coupled with a 6545XT AdvanceBio LC/Q-TOF equipped with a Dual Agilent Jet Stream source. LC separation was obtained on an Agilent AdvanceBio oligonucleotide column with triethylamine and hexafluoroisopropanol as mobile phase additives to enhance separation and ionization in negative mode.

MS only mode was used in TPI workflow while target MS/MS mode was employed for sequence confirmation workflow. Collision energy (CE) were optimized for each precursor mass individually.

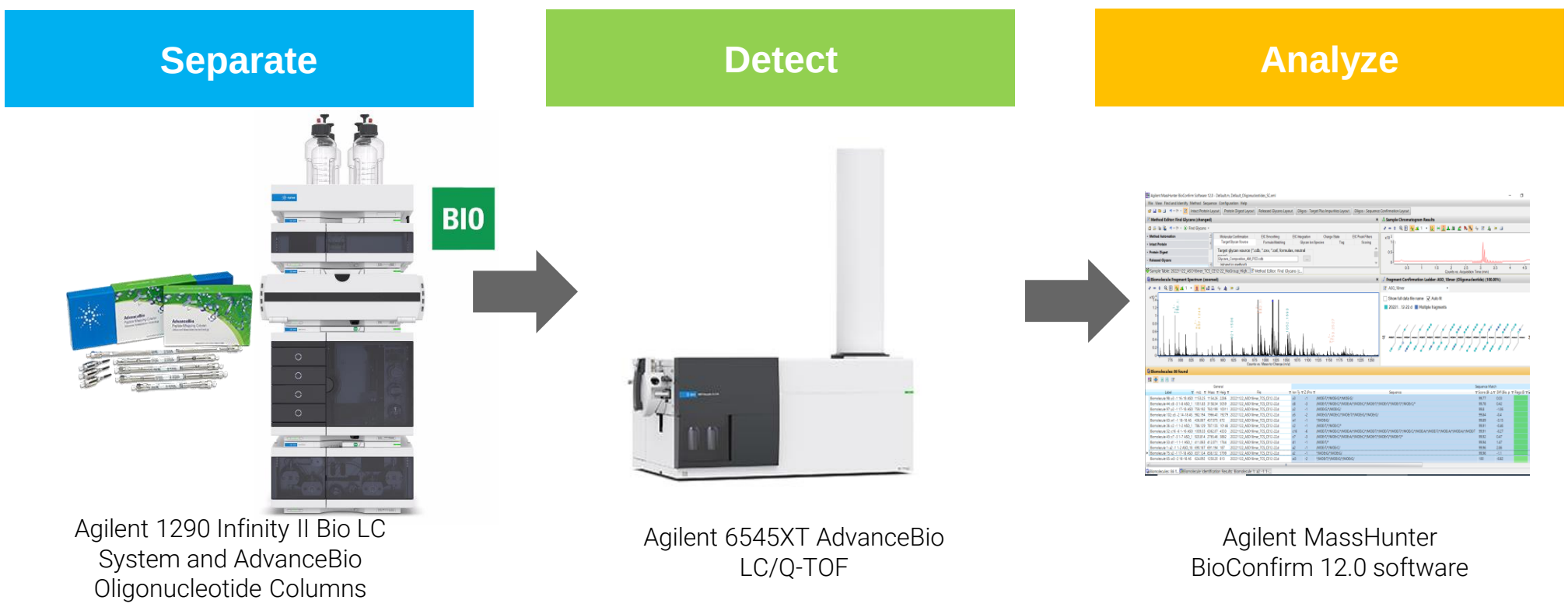

Figure 1. Analytical components of the oligonucleotide characterization for both TPI and SC workflows.

Results and Discussion

Separation of oligonucleotide standard

It is a good practice to run the standards before analyzing the actual samples as system suitability testing of system performance such as resolution and detector sensitivity etc. In this work, Agilent RNA resolution standard was used to check the performance. Excellent chromatographic resolution was achieved among four different lengths of standards as shown in Figure 2.

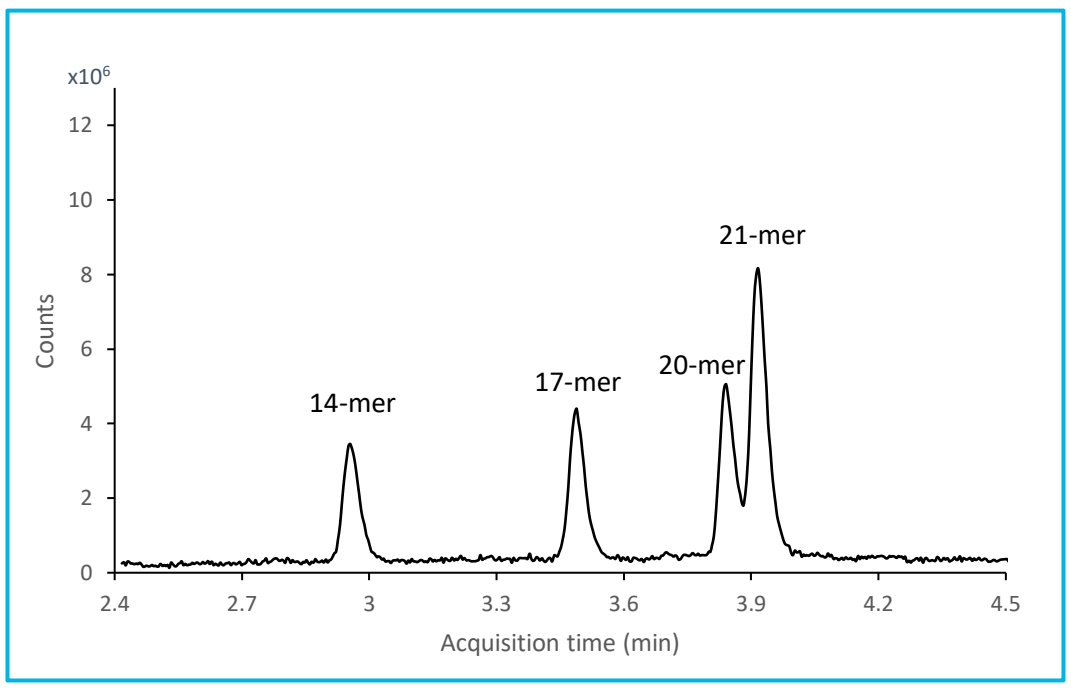

Figure 2. Total ion chromatogram of Agilent RNA Resolution Standard.

Identification of ASO 18-mer with Target Plus Impurities (TPI) workflow

Max Entropy Deconvolution algorithm was used to confirm the identity of an ASO 18-mer sample (average mass 7127.2001 Da). The 10 min LC gradient enabled good separation of the full-length from impurities as shown in Figure 3A. Excellent MS isotopic resolution for the −7 charge state of the oligonucleotide was achieved. Mass accuracy as low as −0.73 ppm was obtained for the ASO peak to confidently confirm the sample identity.

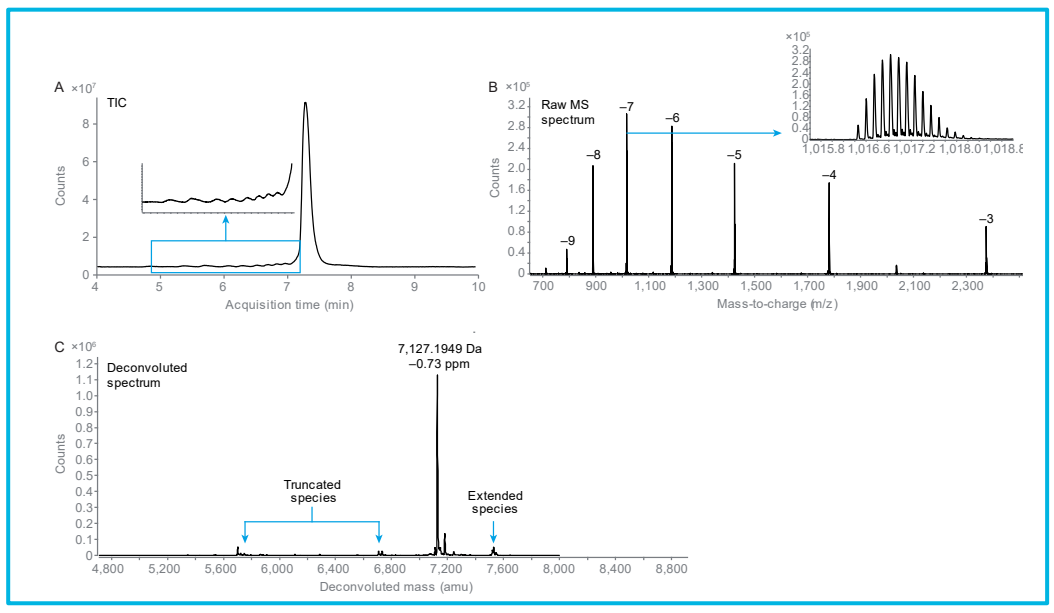

Figure 3. LC/MS analysis of ASO 18-mer.

Relative quantification of 5’-truncated impurities of ASO 18-mer with Target Plus Impurities (TPI) workflow

Find-by-Formula algorithm, a targeted approach, was used in the relative quantification analysis of ASO 5'-truncates with linker.

The series of the impurities distributed across the entire separation (Figure 4). Accurate monoisotopic masses and reproducible relative quantitation results for all targeted impurities were demonstrated in Table 2.

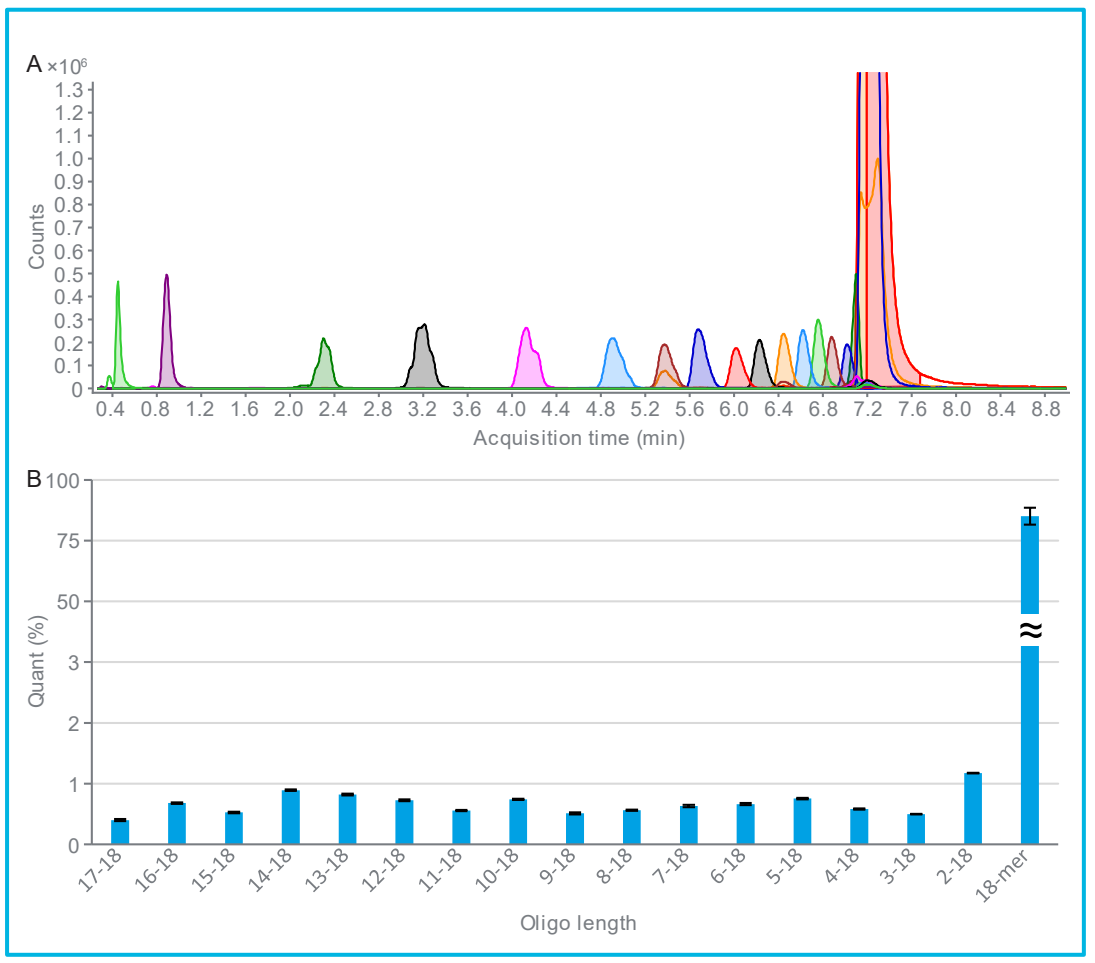

Figure 4. Extracted ion chromatograms (A) and relative quantification results (B) of ASO and its 5’-truncated impurities.

Peak	Oligo Length	RT(min)	Calculated Mono Mass	Measured Mass	Avg Mass Accuracy (ppm) (n=6)	Avg % Quant (n = 6)	Std Dev	RSD(%)
1	17-18	0.45	760.2000	760.2003	0.40	0.40	0.02	4.29
2	16-18	0.90	1154.2600	1154.2605	0.46	0.68	0.01	1.71
3	15-18	2.31	1547.3359	1547.3354	-0.33	0.53	0.01	2.59
4	14-18	3.20	1966.4024	1966.4013	-0.56	0.89	0.01	1.46
5	13-18	4.12	2360.4623	2360.4611	-0.51	0.82	0.01	1.79
6	12-18	4.90	2763.5339	2763.5316	-0.82	0.73	0.02	2.07
7	11-18	5.36	3166.6054	3166.6028	-0.85	0.56	0.01	1.86
8	10-18	5.67	3560.6654	3560.6628	-0.73	0.74	0.01	1.58
9	9-18	6.01	3963.7369	3963.7339	-0.77	0.51	0.02	3.37
10	8-18	6.21	4356.8129	4356.8084	-1.04	0.56	0.01	2.15
11	7-18	6.44	4750.8729	4750.8683	-0.96	0.63	0.02	2.94
12	6-18	6.61	5144.9328	5144.9274	-1.07	0.67	0.02	2.47
13	5-18	6.75	5538.9928	5538.9875	-0.96	0.76	0.01	1.83
14	4-18	6.87	5932.0688	5932.0620	-1.14	0.58	0.01	1.69
15	3-18	7.01	6335.1403	6335.1337	-1.04	0.50	0.01	1.04
16	2-18	7.09	6728.2163	6728.2055	-1.60	1.18	0.01	0.47
Target	18-mer	7.19	7122.2762	7122.2699	-0.89	5.40	0.14	0.16

Table 2. Impurities analysis summary on ASO 18-mer (n=6).

Results and Discussion

Sequence confirmation of oligonucleotides with Sequence Confirmation (SC) workflow

Two synthetic oligonucleotides (ASO 18-mer, aptamer 28-mer) with varied sequence, length, modifications were investigated using the sequence confirmation workflow.

The LC/MS/MS method was optimized for individual oligonucleotides in the aspects of optimal charge state selection, collision energy (CE), and MS/MS acquisition time to obtain the best fragmentation results and highest sequence coverage.

A rapid 5-minute LC gradient method was developed for sequence confirmation workflow. Good MS/MS spectra were obtained for both samples.

For the 2-methoxyethoxylated ASO sample (average mass 7127.2001 Da), most intensive charge state -7 was selected as precursor (*m/z* 1017.0337) and collision energy 15 eV was applied for MS/MS fragmentation. Adequate fragments were generated for sequence confirmation with 100% sequence coverage from just one single injection (Figure 5). Similarly, the 100% sequence coverage of 28-mer aptamer (average mass 9116.5566 Da), which contains fluoro U/T and methylated A/G, was successfully achieved with one injection whereby charge states -10 precursor ion (*m/z* 910.5288) was fragmented at collision energy 18 eV (Figure 6).

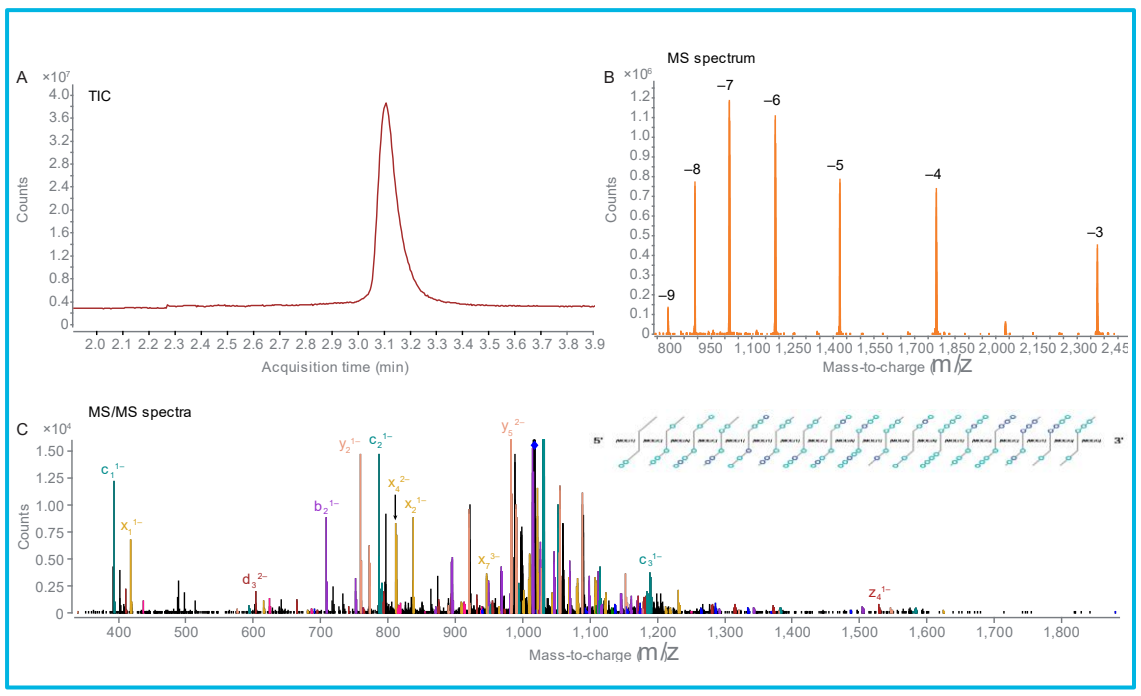

Figure 5. LC/MS/MS analysis of ASO sequence confirmation. (A) total ion chromatogram (TIC) (B) raw MS spectrum (C) MS/MS spectra with fragment confirmation ladder.

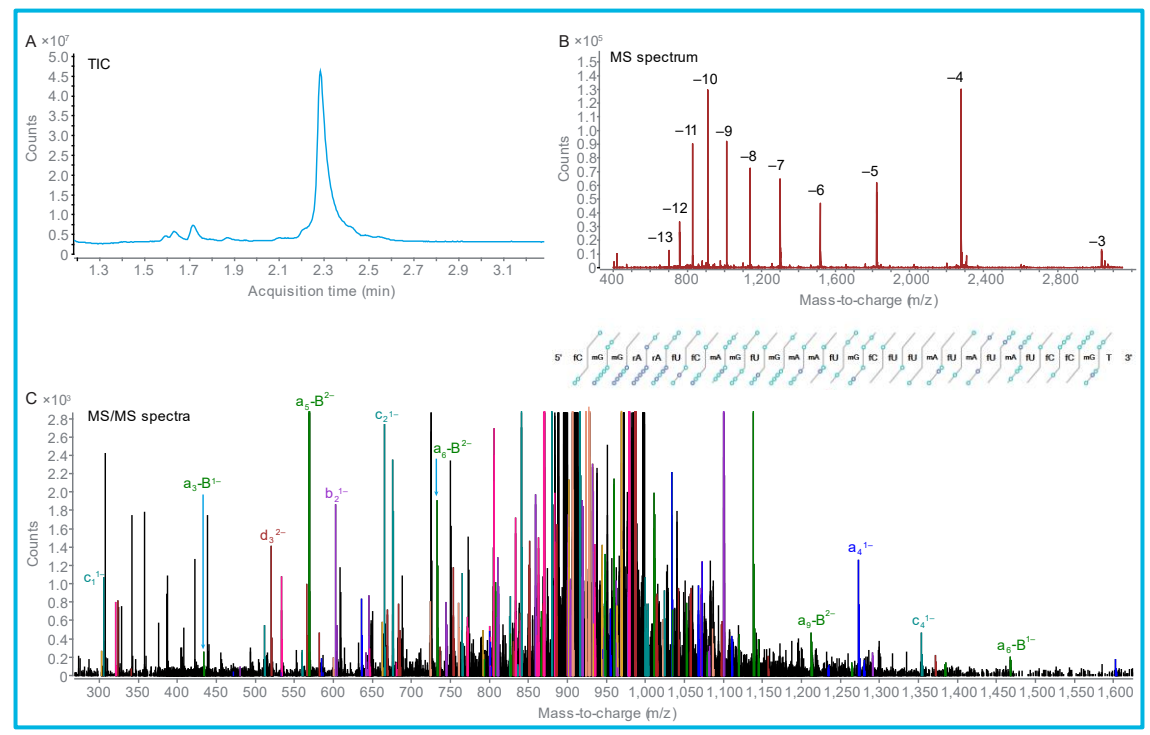

Figure 6. LC/MS/MS analysis of aptamer sequence confirmation. (A) total ion chromatogram (TIC) (B) raw MS spectrum (C) MS/MS spectra with fragment confirmation ladder.

Conclusions

The Agilent 1290 Infinity II Bio LC coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF demonstrated excellent chromatographic separation, reproducible quantification results, and superior LC/MS and HRAM MS/MS data. The two novel Targeted Plus Impurities (TPI) and Sequence Confirmation workflows in the Agilent MassHunter BioConfirm software version 12.0 greatly improve the data processing efficiency of oligonucleotide identity, impurities analysis, and sequence confirmation.

References

[1] Wong, D.; Rye, P. An Integrated Workflow for the Analysis of Oligonucleotides and Their Impurities by Agilent High-Resolution LC/(Q-)TOF Mass Spectrometry. Agilent Technologies application note, publication number 5994-4817EN, 2022.

[2] Wong, D.; Rye, P. An Integrated Comprehensive and Integrated Workflow for Oligonucleotide Sequence Confirmation by Agilent High-Resolution LC/Q-TOF. Agilent Technologies application note, publication number 5994-5071EN, 2022.

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